High-throughput screening of Deubiquitylase enzyme (DUB) activity/specificity and inhibitor screening by MALDI-TOF mass spectrometry

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Introduction

Ubiquitylation regulates a large number of biological processes in eukaryotic cells and it is reversible by specific cleavage through deubiquitylases (DUBs). DUBs are key regulators of the ubiquitin system and play an essential role in cell homeostasis[1]. As DUBs are dysregulated in various diseases, they are considered attractive drug targets for cancer treatment. High-throughput screening (HTS) methods using physiological substrates is important for the identification of novel drug candidates. Several chemical probes[2] as well as fusions of fluorogenic reporters[3,4] to the C-terminal glycine of ubiquitin are widely deployed. However, these methods make use of artificial and non-physiological substrates, thus are not suitable for assessing the linkage specificity of DUBs. As these are artificial substrates do not contain physiological isopeptide bonds, screening assays using these substrates could potentially identify compounds that might not inhibit the deubiquitylation of physiological substrates. It is possible to undertake DUB assays with more physiologically related diubiquitin molecules, however these assays use low-throughput SDS–PAGE methods and require large amounts of enzymes and substrates.

We have developed a new, sensitive, reproducible and robust assay for the analysis of DUB *in vitro* activity and specificity using physiological substrates[5]. We made use of a highly sensitive and fast MALDI-TOF mass spectrometer and MTP AnchorChip 1536 TF target plates that are suitable for robotic automation and thus HTS. Isotopically labeled ubiquitin (^{15}N-Ubiquitin) was used as an internal standard as it guarantees identical extraction, crystallization and gas-phase behaviour and thus highly accurate and precise quantification results[6].

Methods

An utraflextreme MALDI-TOF mass spectrometer (Bruker Daltonics) allowed the analysis of 1536 samples on a single target. Its high resolution and mass accuracy enabled baseline-resolution of the ubiquitin isotopic patterns and thus reliable quantitation of the area under the isotopic distribution. It permitted clear separation of the isotope envelopes of doubly-charged diubiquitin molecule (m/z 8556.64) and the singly-charged monoubiquitin (m/z 8565.76) (Figure 1).

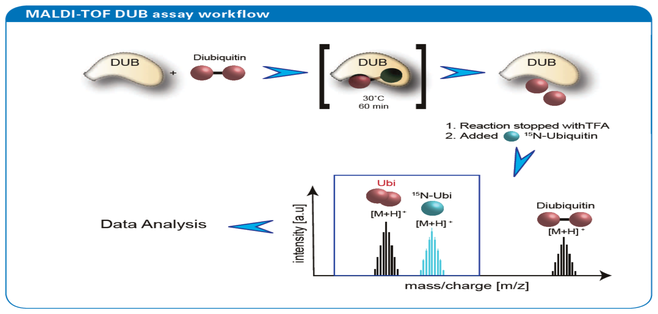

Fig. 1: Diubiquitin is hydrolyzed by DUB to form 2 Ubiquitin (Ubi) molecules. Each of the 42 DUBs expressed and purified in-house is incubated with all 8 Diubiquitin isomers individually (M1, K6, K11, K27, K29, K33, K48 and K63) for 60 min at 30°C. The reaction is stopped and ^{15}N-Ubiquitin is added, which serves as an internal standard. The assay mixture is analyzed by MALDI-TOF and the Ubi/^{15}N-Ubi ratio is used to calculate the consumed Diubiquitin.

All the DUB enzymes were produced in-house and stored in a buffer containing DTT. The eight diubiquitin linkages were purchased from BostonBiochem, whereas DUB inhibitors were either purchased from Tocris Bioscience, Calbiochem/ Merck, Sigma-Aldrich or produced in-house. The DUB assay reaction mixture consisted of recombinant DUB (0.1-1,000 ng), diubiquitin (typically 125 ng (7,300 fmol)) in 40 mM Tris-HCl pH 7.5, 1 mM DTT and 0.005% BSA in a total volume of 5 µl assay volume. Reactions were performed for 1 hour at 30 °C and terminated by addition of 1 µl of 10% (v/v) TFA.

Results

Linearity and reproducibility of the assay were confirmed by analysing standard curves over the ubiquitin concentration range of 10–10,000 nM in the presence of 250 nM 15N-Ubiquitin and 874 nM diubiquitin in three separately performed experiments on different days. Addition of 15N-ubiquitin and/or diubiquitin isomers did not affect sensitivity with which ubiquitin could be detected and quantified (Figure 2a). Average correlation coefficients (r2) for the three curves were not less than 0.99 showing high linearity over a range of more than 500. The mean intraday precision and interday accuracy for ubiquitin/15N-ubiquitin were 8% and 10% respectively. The lower limit of quantitation (LLOQ), defined as the lowest concentration that could be measured with a precision and accuracy better than 20%, was 10 nM (Figure 2b).

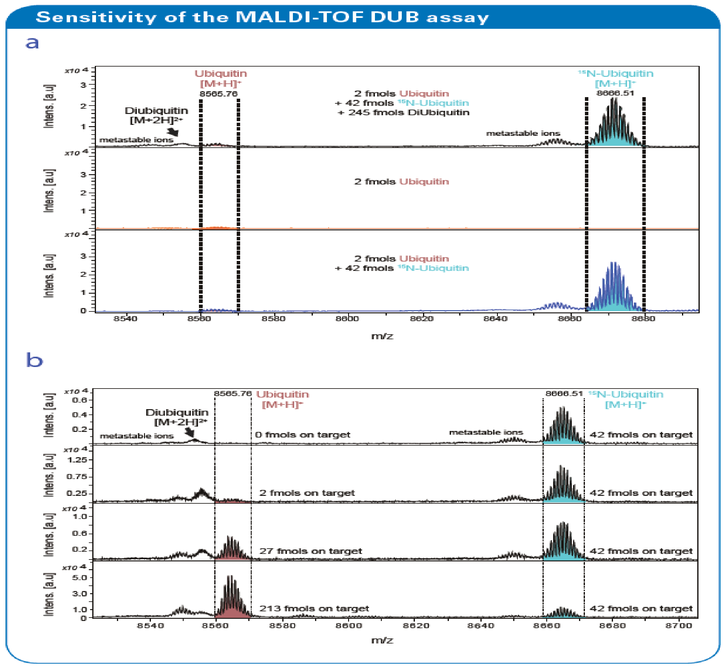

Fig. 2: The MALDI-TOF DUB assay shows high sensitivity.
a) Presence of Diubiquitin [M+2H]$^{2+}$ does not compromise quantification of Ubiquitin MH$^+$.
b) MALDI-TOF spectra for Ubiquitin and ^{15}N-Ubiquitin, in the presence of K11 linked Diubiquitin. The lower limit of detection (LLOD) is 2 fmol of Ubiquitin on the target.

Utilizing the new assay, we assessed the specificity of 32 recombinant human DUBs against all possible ubiquitin chain linkages. This represents almost 50% of the DUBs encoded in the human genome. We determined the DUB activity at five different enzyme concentrations (from 0.02 to 200 ng/µL) against M1/linear, K6, K11, K27, K29, K33, K48 and K63-linked diubiquitin isomers, all at a final concentration of 1.46 µM in the assay. We performed more than 5,520 enzymatic reactions, providing the largest published resource for DUB specificity and activity (Figure 3).

Finally, we evaluated whether the MALDI-TOF DUB assay had potential to assess potency and selectivity of DUB inhibitors. We set up a panel of 32 active DUBs each assayed with the preferred diubiquitin isomers displaying the highest specific DUB activity at the lowest concentration of the related diubiquitin. As proof-of concept, we screened by this method previously reported DUB inhibitors and inhibitor candidates as well as E2/E3-ligase inhibitors that have the potential to alkylate Cys residues against those highly active DUBs from our assay (Figure 4a). In addition, we performed IC50 measurements for the DUBs that were most potently inhibited (Figure 4b). This shows that the MALDI-TOF DUB assay is capable to perform screening of compounds as potential DUB inhibitors.

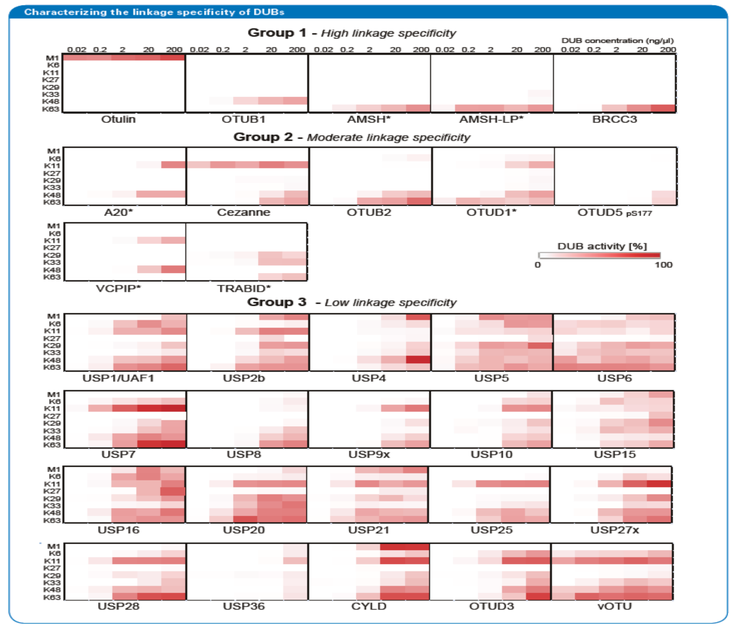

Fig. 3: Increasing concentrations (0.02-200 ng/µl) of DUBs incubated in triplicate with 1.46 µM of Diubiquitin. DUB activity for individual isomers is shown in a gradient of white (0%) to dark red (100%). The DUBs can be grouped into enzymes cleaving specifically one linkage type (group 1), few linkage types (group 2), and unspecific (group 3) presence of K11 linked Diubiquitin.

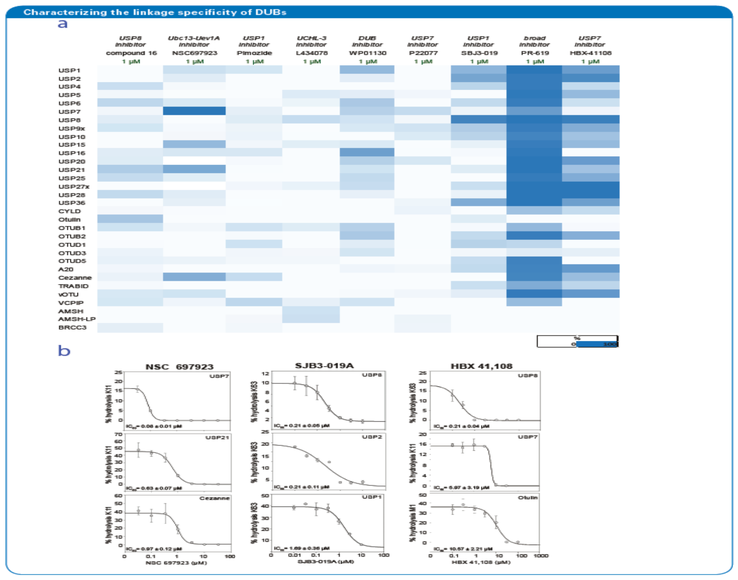

Fig. 4: (a) Nine DUB inhibitors were pre-incubated for 35 min at 1 µM in duplicate with a panel of 32 DUBs and subsequently the specific substrate is added and incubated for 60 min (30°C). Inhibition rates are color-coded with strongest inhibition in dark blue.
(b) A subset of three inhibitors was chosen to characterize in more detail by determining their IC50 for three DUBs.

Summary

We describe a novel screening method to assay DUB activity and specificity that is highly sensitive, reproducible and reliable. It is also able to carry out precise quantitative measurements at a rate of ~5 sec per sample spot. We determined the specificity of 32 active human DUBs, several of which showed high specificity for one single chain type. This allowed us to generate an array of preferred chain types and lowest concentrations of activity for each DUB. Proof-of-concept experiments for nine compounds showed that the assay is a powerful in vitro approach to define the substrate specificity of DUBs and screening of putative specific DUB inhibitors. The assay is easily performed in microtiter plate formats with low µl assay volumes and 1536-spot sample targets that are suitable for automation, thus enabling a fully automated MALDI high-throughput screening system for Drug Discovery.

1. Komander, D. & Rape, M. Annu. Rev. Biochem. 81, 203-229 (2012)
2. Hemelaar, J. et al. Mol. Cell. Biol. 24, 84–95 (2004).
3. Hassiepen, U. et al. Anal. Biochem. 371, 201–207 (2007).
4. Dang, L. C., Melandri, F. D. & Stein, R. L. Biochemistry 37, 1868–1879 (1998).
5. Ritorto, M.S., Ewan, R., Perez-Oliva, A., Knebel, A., Buhrlage, S.J., Wightman, M., Wood, N.T., Gray, N.S., Morrice, N.A., Alessi, D.R., Trost, M. Nature Communications, 5:4763 (2014)
6. Anderson NL, Razavi M, Pearson TW, Kruppa G, Paape R, Suckau D. J Proteome Res.;11(3):1868-78 (2012).